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Light-scattering spectroscopy differentiates fetal from adult nucleated red blood cells: may lead to noninvasive prenatal diagnosis

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Abstract

Present techniques for prenatal diagnosis are invasive and present significant risks of fetal loss. Noninvasive prenatal diagnosis utilizing fetal nucleated red blood cells (fNRBC) circulating in maternal peripheral blood has received attention, since it poses no risk to the fetus. However, because of the failure to find broadly applicable identifiers that can differentiate fetal from adult NRBC, reliable detection of viable fNRBC in amounts sufficient for clinical use remains a challenge. In this Letter we show that fNRBC light-scattering spectroscopic signatures may lead to a clinically useful method of minimally invasive prenatal genetic testing.

The incidence of birth defects in the United States due to chromosomal abnormalities is estimated to be 34,000 per year [1]. Of these, approximately 0.65% cause significant morbidity, and another 0.2% eventually will affect reproduction [1]. Fetuses with aneuploidy, an abnormal number of chromosomes, account for 6% to 11% of all stillbirths and neonatal deaths [2]. The most common form of aneuploidy at birth (1:800–1:1000 live births) is Down syndrome or trisomy 21 (three copies of chromosome 21). Aside from Down syndrome, other autosomal trisomies, sex chromosome abnormalities, and structural balanced and unbalanced abnormalities can be associated with high neonatal mortality and both short- and long-term morbidity. The risk of having a child with aneuploidy increases with increasing maternal age. Prenatal screening or diagnostic testing for chromosomal abnormality in the fetus or embryo carried out early in pregnancy, combined with appropriate genetic counseling, can help parents make informed decisions about the pregnancy and neonatal care. Currently, prenatal diagnosis of aneuploidy relies on fetal cells obtained by amniocentesis, chorionic villus sampling, or percutaneous umbilical blood sampling. It is assumed approximately 9% of all deliveries every year in the United States involve midtrimester amniocentesis [3]. These are invasive procedures presenting significant

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risk to the fetus. Alternatively, noninvasive methods to obtain fetal cells or free fetal DNA circulating in maternal peripheral blood during pregnancy have received much attention [4,5].

Fetal nucleated red blood cells (fNRBC) have generated the greatest interest for prenatal diagnosis [6–9]. They present a full complement of genes, possess morphologic characteristics that distinguish them from other nucleated cells [10], and with a limited life span of about 25 to 35 days, it is unlikely that fNRBC could persist from one pregnancy to the next [11]. While NRBC are extremely rare in normal adult circulation, fNRBC do make up a small fraction (perhaps $1/10^9$) of circulating cells during pregnancy. Some success in isolating fNRBC from the maternal circulation has been realized (Bianchi *et al.* [12] isolated approximately one cell per milliliter of maternal blood when the karyotype is normal), but current methods to identify, isolate, and analyze fNRBC generally have been found to be clinically unsuitable for prenatal diagnosis [5].

Established methods can be used to separate NRBC from other cells in maternal circulation. However, owing to very low concentration in the maternal blood (1 per 10^9 cells) and interference by maternal, adult nucleated red blood cells (aNRBC) [13] present at a similar concentration (1 fNRBC to 1–100 aNRBC), reliable detection of fNRBC [14] and isolation from aNRBC for clinical use remains a challenge. For example, chromatographic methods risk cell damage and loss of genetic material, and current cell sorting methods require tagging, which interferes with and/or limits the applicability of available diagnostic analyses. In this Letter we use light-scattering spectroscopy (LSS) to address the unsolved problem of differentiating fNRBC from aNRBC.

Recently we developed biomedical LSS and showed its unique ability to optically probe nuclear morphology without damaging cells [15]. Since fNRBC differ from aNRBC in nuclear morphology [10], this approach may lead to a robust optical technique to enable prenatal genetic testing based on enrichment and recovery of intact fNRBC from maternal peripheral blood.

We performed experiments with fNRBC from fetal umbilical cord blood and aNRBC from adult female bone marrow. The protocol was reviewed by the Institutional Review Board of Beth Israel Deaconess Medical Center, and the requisite approvals were obtained. Standard immunofluorescence techniques were used to distinguish NRBC in each of the following specimens.

To identify fNRBC in umbilical cord blood, we used two different monoclonal antibody techniques, as no available technique is 100% specific. First, we used mouse monoclonal antibody (MAB 3432, Chemicon, Temecula, Calif.) directed against glycophorin A, an erythrocyte-specific cell surface antigen. As the secondary antibody we used a red fluorescence marker, phycoerythrin (PE) conjugated goat antimouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.). These cells were then double stained by incubating with green fluorescence DNA specific dye Syto 16 (Molecular Probes, Inc. Carlsbad, Calif.), which stains the nucleus. Using this technique, the nucleated red blood cells were double stained with both green and red fluorescers [Fig 1(a)].

Second, we used a mouse monoclonal antibody directed against human erythroid cell surface antigen HAE9 (MAB 2115, Chemicon, Temecula, Calif.), which recognized only NRBC. Green fluorescent isothiocyanate (FITC) conjugated antimouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) was used as the secondary antibody [Fig. 1(b)].

To obtain aNRBC from bone marrow specimens from adult females, we used monoclonal antibody HAE9 as the primary antibody with Alexa Fluor 488 (Molecular Probes, Inc., Carlsbad, Calif.) conjugated goat antimouse IgM as the secondary antibody.

Cells successfully identified by the above techniques were studied with our recently developed Confocal Light Absorption and Scattering Spectroscopic (CLASS) microscopy system [16], which combines confocal microscopy with LSS, to obtain the light-scattering spectra of the immunostained cells.

The CLASS image of aNRBC and spectra of their various subcellular compartments are presented in Fig. 2. Each compartment of the aNRBC has an informative spectrum. For example, Fig. 2(a) exhibits an oscillatory structure of approximately 30 nm period, characteristic of light scattering from a large particle such as the nucleus. The spectrum in Fig. 2(b), which is taken close to the nucleus, in addition to the high-frequency oscillations, also exhibits a low-frequency oscillation characteristic of smaller scatterers, such as submicron organelles (about 300 nm period). In Fig. 2(c), the scattering signatures are almost gone, but the hemoglobin absorption bands at 540 nm and 580 nm, present in all three compartments, are clear.

Typical CLASS spectra of fetal and adult NRBC are presented in Fig. 3. For a given cell, spectra were acquired by scanning the entire cell point by point and summing the spectra together. These summed spectra were averaged for three to four cells of each type, and the mean spectra were plotted in Fig. 3. Error bars show the standard deviation. Light scattering spectra clearly differentiate fetal from adult NRBC in the spectral region from 600 to 780 nm, where the standard deviations do not overlap. The p value for a Student's t test using the intensities at 780 nm is $p=0.0004$, which is statistically significant. This is the same as using the slopes of the spectra.

Spectra of NRBC of both fetal and adult bone marrow origin are dominated by oscillatory behavior characteristic of light scattering by the nucleus [16]. Overlap of the light-scattering spectra of the cell nucleus and other cell compartments also contributes to the oscillatory features seen in Fig. 3. While the reported spectra were acquired with CLASS, it is important to note that the summed, whole-cell spectra differentiate fNRBC from aNRBC, so confocal, intracellular spatial resolution, which might slow potential screening and harvesting protocols, is not required.

We note that the presence of the immunofluorescence markers should not interfere significantly with the light-scattering spectra of the NRBC. First, the fluorescence emission produced under the conditions of the light-scattering measurement is very weak relative to the elastically scattered light. Second, the fluorescence band structure is narrow (25 nm to 50 nm) compared with the scattering bandwidth (~400 nm) and broad compared with the higher

frequency oscillations (~10 nm) of the light-scattering spectra. Finally, the dimensions of the markers are on the order of 10 nm, well below the light-scattering wavelengths (500 nm to 900 nm). Therefore, alteration of the inherent light-scattering properties of the labeled cells compared with native cells is negligible and does not affect the conclusion drawn here. Of course, a working clinical system will not require fluorescence markers.

The light-scattering spectra of fNRBC and aNRBC are clearly distinguishable, and the standard deviations do not overlap at wavelengths longer than 600 nm. The distinctions between the spectra of these two cell types arise from differences in morphology and biochemistry. While morphological differences are not readily apparent in visual images obtained with conventional microscopy techniques, light-scattering spectra respond sensitively to small differences in nuclear size, nuclear volume to cell volume ratio, and organelle size and number density distributions [15,16], resulting in the reported differences in the light-scattering spectra of fNRBC and aNRBC. Identification of fNRBC in maternal circulation using LSS has the potential to yield fNRBC with minimal damage, thereby providing useful fetal genetic material for prenatal diagnosis.

The results we report here indicate that CLASS is capable of reliably distinguishing fetal NRBC from adult NRBC without tagging, fixation, or risk of cell damage. In addition, because sample preparation is minimal for CLASS or LSS measurements, multistage preparation procedures that result in cumulative cell loss are eliminated. There may be a wealth of informative cell biology and chemistry revealed by analysis of the detailed structure of fNRBC light-scattering spectra, some of which may itself be of value for prenatal diagnosis. Nonetheless, the qualitative differences in the overall spectra of fNRBC and aNRBC presented above indicate LSS may provide a useful technique for developing a clinical method capable of recovering sufficient fNRBC from peripheral maternal blood for minimally invasive prenatal genetic testing.

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Fig. 1.
(Color online) Individual cells in an umbilical cord blood sample. (a) MAB-PE-Syto 16 stained cells, (b) MAB-FITC stained cells.

Fig. 2.
 (Color online) CLASS image and spectra of the individual subcellular compartments of adult bone marrow NRBC: (a) nucleus, (b) smaller organelle near nucleus (dashed curve, oscillating with low frequency, is provided to guide the eye), (c) compartment containing only hemoglobin.

Fig. 3.

(Color online) Fetal and adult NRBC have distinctly different spectra. The upper curve is the mean LSS spectrum of fNRBC, and the lower curve is the mean LSS spectrum of aNRBC.

Bars are the standard deviation of measurements taken on multiple cells.